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Population Genetic Analysis of the Southwestern Willow Flycatcher: 1996 -1997

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Executive Summary

We conducted a molecular genetic analysis of willow flycatchers (*Empidonax traillii*), with an emphasis on the endangered southwestern subspecies (*E.t. extimus*). This subspecies has a small estimated population size (approximately 500 breeding pairs), and breeds primarily in small groups (fewer than 5 territories) and at scattered locations. Our genetic study was designed to address concerns regarding the level of genetic variation present within the overall population and among different breeding groups.

The objectives of our genetic analysis were: (1) determine the level of genetic variation present within and among flycatcher breeding groups; (2) determine whether flycatcher breeding groups are reproductively isolated or if there is evidence of substantial gene flow between groups; and (3) find out which breeding groups are most closely related (share the greatest degree of genetic similarity).

We collected blood samples from willow flycatchers that were live-captured in mist-nets at numerous breeding sites in Arizona and Colorado, as well as a few sites in California, Nevada, and New Mexico. Blood was obtained by clipping off a portion of the toenail of each flycatcher, and placing the resulting drop of blood in buffer. Blood samples were kept cool until returned to the laboratory. DNA was extracted from blood using standard DNA extraction protocols. Polymerase chain reaction (PCR) techniques were used to conduct Amplified Fragment Length Polymorphism (AFLP) analysis of nuclear DNA, and DNA sequencing of the cytochrome b region of the mitochondrial DNA.

AFLP analysis resulted in unique DNA fingerprints for all individuals, and showed minor but statistically significant genetic variation between some breeding sites. However, the overall genetic differences are minor between sites as compared to within sites, and may occur primarily because most individual willow flycatchers show some degree of fidelity to their natal and breeding sites. The genetic patterns demonstrate that there is a substantial amount of genetic exchange among flycatcher breeding groups, both within and between drainages. Thus, southwestern willow flycatchers breeding groups appear to be functioning as subpopulations within a larger metapopulation. AFLP results also suggest that the current southwestern willow flycatcher population does not appear to suffer from low overall genetic diversity.

Analysis of mitochondrial DNA sequences from Arizona revealed the presence of three distinct mitochondrial lineages. Two of these were distributed widely across the low and mid-elevation sites. The third mito-type was found in higher elevation sites and along the Colorado River in the upper Grand Canyon. These three sequences differed from each other by only one or two base pairs, suggesting possible recent divergence and limited differentiation. These mitochondrial patterns do not at this time provide any insight into the subspecific taxonomy of the flycatcher, and can not be used to infer that the third mito-type represents a different subspecies. Sequencing of additional samples from throughout the willow flycatcher's range will be needed before any conclusions can be drawn regarding flycatcher taxonomy or subspecies range boundaries.

From a conservation and management perspective, we did not find any flycatcher breeding groups with substantially unique genotypes or limited genetic diversity. This is consistent with a metapopulation structure with regular genetic exchange among breeding groups via dispersal and movement of individuals among sites. This concurs with recent banding data that show movement of individuals among breeding sites both within and between river drainages.

A metapopulation structure with regular genetic exchange and resultant low differentiation among sites is a desirable condition in that it increases effective population size, reduces the probability of inbreeding depression, and reduces the genetic consequences of the loss of any one population.

The maintenance of this population structure is dependent upon the existence of a number of sites geographically distributed across the bird's range, each large enough to minimize the danger of loss due to stochastic events and close enough to allow for frequent genetic exchange. Another important consideration is the protection of flycatcher breeding areas with high productivity and breeding success. This would increase both the number of individuals in the overall population (hence reducing the potential for extinction) and the number available for dispersal among sites (which increases genetic interchange and maintains genetic diversity).

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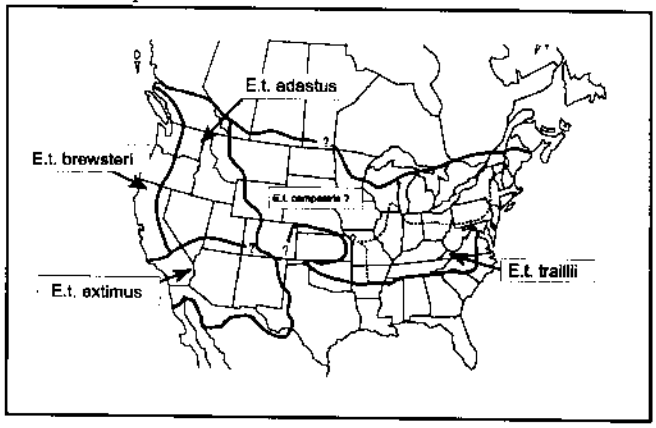
Introduction

The southwestern willow flycatcher (*Empidonax traillii extimus*) is a federal endangered species (USFWS 1995) that breeds at scattered, isolated riparian sites throughout it range (Unitt 1987, Hubbard 1987, Maynard 1995, Cooper 1997, Owen and Sogge 1997, Sferra et al. 1997, Marshall *in review*). It is one of four or five willow flycatcher subspecies breeding widely across most of the lower 48 states (Unitt 1987, Browning 1983; Figure 1). When proposing the flycatcher for listing and in subsequent biological opinions, the U.S. Fish and Wildlife Service (USFWS 1993, 1997) identified reduced gene flow between these isolated breeding populations as a potential threat to the species.

The nature and degree of genetic diversity remaining within the overall population, and within and between breeding groups, is a significant factor with regard to the status, conservation, and management of the southwestern willow flycatcher. Preservation of genetic variability is important in that genetic diversity may be correlated with productivity, the presence and/or frequency of detrimental alleles, and the ability to adapt to future environmental changes (Nevo 1978, Weins et al. 1989, Seitz and Loeschcke 1991).

Figure 1. The breeding range distribution of the subspecies of the willow flycatcher (*Empidonax traillii*). Based on Unitt (1987) and Browning (1993), with modifications based on unpublished USFWS data.



Differences in genetic variability can also tell us whether the flycatchers at the different breeding sites are genetically isolated (and hence distinct subpopulations), or whether there is genetic mixing between the breeding sites. Genetically fragmented and isolated populations may have to be managed specifically with those characteristics in mind. As with most bird species, the willow flycatcher has the potential to disperse long distances and to move among different breeding sites within or between years. Such dispersal and movements would reduce the degree of reproductive and genetic isolation, and increase overall genetic diversity. Prior to the onset of this study (which includes the banding and demographic components as reported in Paxton and Sogge 1996, and Paxton et al. 1997), there was no information on the nature and degree of such movements, and therefore whether populations would be expected to show high levels of genetic and reproductive isolation.

Population genetic data can also identify the overall level of genetic variation present. Genetic techniques can also determine which breeding groups are most closely related, identify breeding groups with unique genotypes, and model whether genetic diversity is best maintained by focusing on habitat conservation and enhancement efforts at one or more larger populations, versus several smaller breeding groups.

Because of these considerations, it was important to conduct a molecular genetic analysis of the southwestern willow flycatcher. Such genetic analyses have been successfully used to provide information on taxonomy, genetic variation, historical patterns of population fragmentation, hybridization, and population structure (Avise 1994, Zink et al. 1995), as well as for conservation planning (Lande 1988). Accurate information on the level and pattern of genetic variation present within the subspecies and specific populations will allow land and resource managers to evaluate the effects of population reduction and fragmentation, which may be critical concerns for the long-term

viability of this species.

The objectives of our genetic analysis were:

> 1 - determine the level of genetic variation present within and among flycatcher breeding groups;
>
> 2 - determine whether flycatcher breeding groups are reproductively isolated or if there is evidence of substantial gene flow between groups;
>
> 3 - find out which breeding groups are most closely related (share the greatest degree of genetic similarity)

This work was originally funded by the Arizona Game and Fish (AGFD) Department Heritage Fund, and the U.S. Bureau of Reclamation (Bureau) Phoenix Area Office. Based on research contracts with AGFD and the Bureau, our initial focus was collecting and analyzing data from Arizona, with emphasis on the Roosevelt Lake and San Pedro River populations. However, with subsequent funding from the U.S. Geological Survey Biological Resources Division and the Bureau's Salt Lake City office, we were able to collect and analyze willow flycatcher genetic samples from sites in Colorado, Nevada, and California. Including genetic data from flycatchers throughout the southwest provides a greater understanding of the overall patterns and diversity than would be possible based on fewer sites or a more limited geographic distribution.

There are numerous genetic analyses that can be conducted on a species, and there are a great many important and useful genetic questions that remain to be answered regarding the southwestern willow flycatcher (see Avise in Meffe and Carroll 1997). However, different questions often require different patterns of data collection and/or different genetic analysis techniques. One very important genetic question that is not addressed in our project objectives is that of the nature and geographic distribution of the different willow flycatcher subspecies. In order to answer such taxonomic questions, it would be necessary to collect and analyze DNA samples from populations of willow flycatchers throughout its breeding range. To date, we have received funding only to gather data from within and near the proposed boundary of the southwestern race. The samples that we collected will comprise a critical subset of this rangewide data set, should future funding become available for additional DNA collection and analysis.

Methods

This genetic project is a component of a broader flycatcher banding and demographics research program coordinated by the Colorado Plateau Field Station. It provides complimentary information for understanding population processes and evaluating population viability of the southwestern willow flycatcher. The field work necessary for both components requires the live-capture of territorial and breeding flycatchers. Thus, including these two components in one project was a cost-effective and efficient use of time, personnel, skills, and funding.

We coordinated our efforts closely with AGFD and other researchers working on specific breeding sites throughout the southwest in order to direct our capture efforts. It is only through the assistance of many agencies and persons (see the Acknowledgments section) that we were able to collect samples from so many and so diverse sites.

Flycatcher capture

All adult willow flycatchers were captured using mist nets in a standard method (see Ralph et al. 1993). The mist nets were typically set up in a known breeding territory, and recordings of willow flycatcher vocalizations (both songs and calls) were broadcast from a tape player or compact disk player to attract territorial flycatchers. Often, an *Empidonax* flycatcher decoy was used to compliment the vocalizations. Each captured adult was banded with a uniquely numbered USFWS aluminum band and at some sites a unique combination of color bands (refer to Paxton and Sogge 1996, Whitfield and Enos 1996, Langridge and Sogge 1997, Owen and Sogge 1997, Paxton et al. 1997, Whitfield et al. 1997).

For the genetic analyses presented in this report, we used DNA samples only from breeding or territorial adult flycatchers. To assure that no migrants were included in the samples, we included only adults that were captured during the non-migrant period (June 15 - July 20; Unitt 1987, Sogge et al. 1997), or that were recaptured or resighted at our study sites subsequent to the original capture. Nestlings were not included because they are closely related to each other and do not represent independent genetic samples. Inclusion of nestlings would also artificially raise our estimates of genetic differentiation of breeding sites.

Blood collection

A toenail-clipping technique was used to collect blood from birds in the field. Using nail clippers, one toenail was clipped at the vascularized quick, and the resulting drop of blood was rinsed into a 1.5 ml tube using 10-100 μl of blood collection buffer (1xSSC, 50 mM EDTA). Samples were kept on ice until they could be stored frozen.

DNA isolation

DNA was isolated from blood using a modification of the procedure described by Mullenbach et al. (1989). The tubes containing blood received an appropriate volume (370-470 μl) of blood lysis buffer (10 mM Tris, 1 mM EDTA, 1% SDS, 100 mM NaCl) to achieve a volume of 480 μl. Also, 10 μl of 10 mg/ml Proteinase K and 10 ul of 0.1 M DTT were added (final V = 500 μl). The samples were incubated overnight at 55° C. The following day, 214.5 μl of 5 M NaCl was added, followed by a 30 min incubation at 55° C. The samples were then cooled to room temperature, given an equal volume of chloroform, and vortexed briefly. This was followed by centrifugation for 10 min at 12,000-x g, after which the aqueous-phase supernatants were transferred to a fresh 1.5 ml tube. An equal volume of 100% isopropanol was added, and samples were placed on an orbital shaker for 30 minutes while the DNA precipitated. Then the samples were centrifuged for 30 min. at 12,000 x g and the isopropanol was removed from the pellets. The pellets were washed once with 70% ethanol and centrifuged at 12,000 x g, then dried in a spin-vac

and resuspended in 50-200 ul of TE (10mM Tris, 1mM EDTA, pH 8.0). DNA was electrophoresed on a 0.7% agarose gel to assess template quality and quantity.

AFLP procedure

Genetic markers were generated using the AFLP procedure of Vos et al. (1995) with modifications described below. Restriction-ligation reactions were conducted using 50-200 ng of DNA in 1xRL buffer (10 mM Tris-base, 10 mM K-acetate, 10 mM Mg-acetate, 5 mM DTT, pH 7.5) with 5U of both *Eco*RI (Gibco BRL) and *Mse*I (NEB) restriction enzymes. RL's were incubated for 1 hr at 37°C, at which time the following ligation reagents were added: 5 pmol each of two *Eco*RI adapters (Keygene adapters 91M35 and 91M36), 50 pmol each of two *Mse*I adapters (Keygene adapters 92A18 and 92A19), 1xRL buffer, 0.2 mM ATP, and 1U T4 DNA Ligase. RL's were incubated for an additional 3 hr at 37° C and afterward stored at -20° C. An aliquot of each sample was diluted 1:10 in Te (10 mM Tris, 0.1 mM EDTA, pH 8.0) for use as template in the first selective amplification.

The first and second selective amplifications and polyacrylamide gel electrophoresis took place according to Vos et al. (1995) with modifications described in Travis et al. (1996). Adenine was used as the selective nucleotide in the first amplification. The nucleotides for the second selective amplification are listed in Table 2.

Polymorphic markers were scored manually for each sample. Markers were chosen if they could be scored confidently in a large percentage of all observations. Of the 45 markers chosen for analysis, 44 were scored unambiguously in 97.5% of the observations and 1 marker was scored unambiguously in 93.5% of the observations. Information that could not be scored confidently was entered as missing data.

cytochrome-B sequencing

We selected DNA samples for cytochrome-b sequencing in order to: (1) characterize broad genetic structuring and variation across Arizona, and (2) evaluate variation within one site (Roosevelt Lake).

The entire (1143 bp) cytochrome-b gene was amplified from DNA extracts using Polymerase Chain Reaction (PCR) and primers obtained from Helm-Bychowski and Cracraft (1993; L14827:5' CCACACTCCACACAGGCCTAATTAA-3', H16065: 5'-GGAGTCTTCAGT-CTCTGGTTTACAAGAC-3'). The PCR reaction conditions consisted of 50 ng of DNA, 1x PCR buffer, 3 mM $MgCl_2$, 200 μM of dNTPs, and 1 μM each of primer, and 1 U of *Taq* DNA polymerase. The PCR reaction was performed on an MJ Research MiniCycler thermal cycler machine with the following cycling parameters: 30 seconds at 94° C to denature, 30 seconds at 55° C for annealing, and 2 minutes at 72° C for extension, 35 cycles.

PCR products were concentrated using a Qiagen QIAquick PCR purification kit following the company's instructions with the addition that the PCR reaction's pH was brought down to ~ 6.5 pH for better bonding of DNA to filter. The purified product was visualized using electrophoresis to determine quantity of DNA for each sample. The gene was sequenced using the dye-nucleotide termination method with an ABI 377 DNA sequencer. Sequence obtained from the ABI prism sequencer was then aligned manually and edited with the aid of Sequence Navigator version 1.0.1 (Applied Biosystems).

Data analysis

Separating data for each year: When conducting a population genetic analysis, the resulting information provides a glimpse at the extent of within and between-population diversity at a single point in time. In this study, data were collected at sites over a 2 year period, with many of the sites sampled in both years. Willow flycatchers have been observed to change breeding sites between years (Paxton et al. 1997). For this reason, it was deemed unwise to pool data across years because birds sampled from one site in 1996 frequently were not found at the same site the following year. Dispersing birds may actually have moved to another breeding location the following year. Thus a bird may not have been temporally part of the same population each year, and may have been part of two different populations in successive years. To account for this, we constructed separate data sets for birds collected during each year of this

study. This approach provided us with the advantage of having two independent data sets from which inferences could be made.

Genetic differentiation: We used the program MANTEL-STRUCT (Miller, *in press*) to characterize relationships of individuals by calculating average within and between-site similarities as quantified by the Jaccard coefficient (Jaccard 1908). A graphical representation of average between-site similarities was obtained using the UPGMA cluster analysis feature of NTSYS-pc (Rohlf 1993). The MANTEL-STRUCT program was also used to perform a variation of a Mantel test (Miller *in press*, Sokal and Rohlf 1995) to test the null hypothesis that there were no differences in within and between-site similarities of individuals, which would indicate a lack of structure among Southwestern Flycatcher populations. This analysis was conducted on individuals from all sites simultaneously by calculating the correlation between the inter-individual similarity coefficient matrix and a congruent binary matrix containing 1s in the positions of within-site similarities and 0s in the positions of between-site similarities. In such an analysis, larger correlation coefficients indicate greater genetic differentiation of sites. The significance of the correlation was obtained for the simultaneous analysis of all sites using an asymptotic approximation provided by Mantel (1967).

In addition, we also calculated two more common indicators of genetic differentiation: Θ (Weir 1996, Weir and Cockerham 1984) and Φ_{ST} (Excoffier et al. 1992). Θ was calculated using the Tools for Population Genetic Analyses software package (Miller 1997). Use of this procedure on the presumed dominant AFLP markers generated in this study required the assumption that each marker corresponded to an independently-segregating Mendelian locus whose genotype frequencies corresponded to Hardy-Weinberg equilibrium. Based on these assumptions, TFPGA estimated allele frequencies using the Taylor expansion approach of Lynch and Milligan (1994) and calculated Θ under the assumption of random mating (Weir and Cockerham 1984). We tested the significance of Θ by generating 95% confidence intervals around the statistic through the use of a bootstrapping procedure (5000 replicates). Confidence limits around Θ that were non-overlapping with 0 were taken as evidence for significant genetic differentiation of sites.

We used an Analysis of Molecular Variance (AMOVA) to obtain Φ_{ST} estimates, which were calculated using AMOVA 1.55 (Excoffier 1993). Data files used in the analysis were prepared from our raw data with AMOVA-PREP 1.01 (Miller 1997) using the Euclidean distance metric of Excoffier et al. (1992). Because use of the AMOVA procedure requires the unambiguous distinction of marker profiles, these analyses were performed on slightly smaller data sets containing no missing data (Table 1). The significance of Φ_{ST} was evaluated with an Monte-Carlo procedure consisting of 9,999 replicates.

Table 1. Number of birds sampled from each site during each year. Numbers in parentheses are sample sizes used in AMOVA analyses.

Site Code	Site Name	State	Sample Sizes 1996	1997
ALPI	Alpine, San Francisco River	AZ	4 (0)	-
ALWR	Alamosa National Wildlife Refuge, upper Rio Grande	CO	5 (5)	11 (10)
ARAP	Arapahoe National Wildlife Refuge	CO	-	5 (5)
BCCO	Beaver Creek	CO	-	6 (6)
CAVE	Camp Verde, Verde River	AZ	6 (5)	15 (15)
CBCR	CB Crossing, San Pedro River	AZ	-	5 (0)
CCCO	Clear Creek	CO	-	8 (8)
COOK	Cook's Lake and Cook's Seep	AZ	11 (10)	8 (8)
ESCA	Escalante State Wildlife Area	CO	6 (5)	6 (6)
ESCA	Escalante State Wildlife Area	CO	6 (5)	-
GILA	Gila River near Safford	AZ	6 (6)	-
GREE	Greer at River Reservoir, Little Colorado River	AZ	8 (6)	7 (7)
INHI	Indian Hills, San Pedro River	AZ	-	17 (16)
KERN	Kern River near Isabella Reservoir	CA	12 (11)	24 (24)
KRNY	Kearny, Gila River	AZ	-	8 (7)
MSCP	McIntyre Springs	CO	-	6 (6)
PARA	Pahranagat National Wildlife Refuge	NV	-	6 (6)
PZRA	PZ Ranch, San Pedro River	AZ	20 (17)	6 (6)
RICO	Rio Blanco Lake	CO	-	10 (9)
SALT	Salt River inflow - Roosevelt Lake	AZ	18 (12)	22 (20)
SAYE	Santa Ynez River	CA	-	9 (9)
TONT	Tonto Creek inflow - Roosevelt Lake	AZ	14 (9)	12 (12)
ZUNI	Zuni Pueblo	NM	-	5 (5)
TOTAL			110 (86)	196 (185)

Results

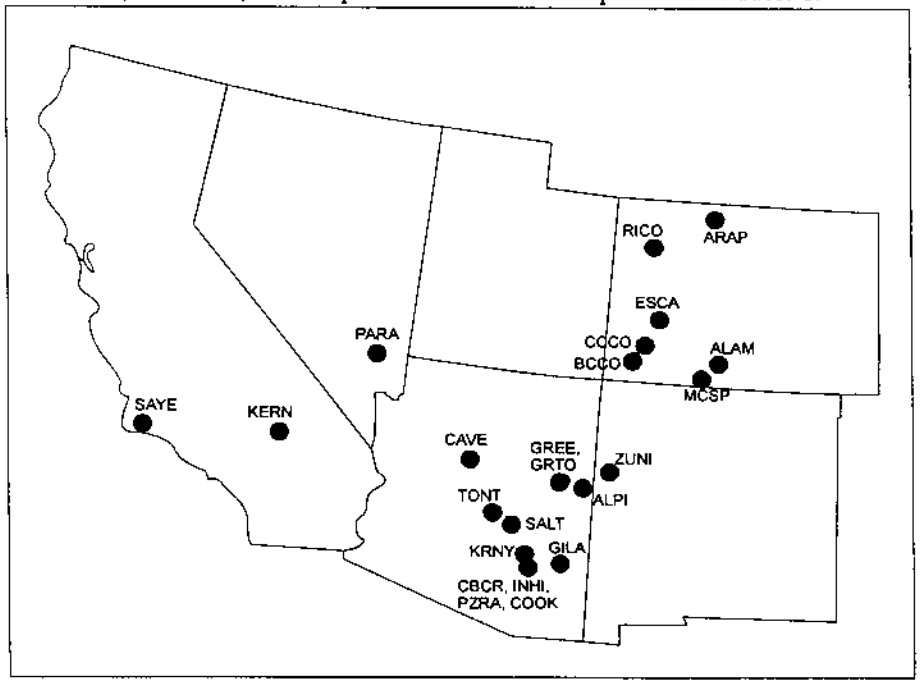
Capture locations

We obtained a sufficient number of blood samples for the AFLP analyses from willow flycatchers at two sites in California, one in Nevada, seven in Colorado, one in New Mexico, and 12 in Arizona (Figure 2). The sites spans a geographic distance of approximately 1,200 km from east to west, and 900 km north to south. Elevation at sites ranged from approximately 30 m (Santa Ynez River, CA) to 2,300 m above sea level (Clear Creek, CO). The type of flycatcher breeding habitat also varied across these sites, ranging from monotypic native vegetation (primarily willow) to monotypic tamarisk, with several sites comprised of a mixture of exotics and natives. Estimated number of breeding territories per site (based on Langridge and Sogge 1997b, Sferra et al. 1997, Owen and Sogge 1997, Whitfield et al. 1997, and USFWS data) ranged from 3 to 34 (Appendix 1).

AFLP Markers

Table 2 lists the total number of monomorphic and polymorphic AFLP markers. Only 45 of 197 polymorphic loci (23%) were actually used in the analysis. The remaining 152 markers were polymorphic but could not be scored with confidence. The number of scorable polymorphic markers ranged from 2 to 14 bands per primer combination.

Figure 2. Approximate locations of sites where willow flycatcher blood samples were obtained and used in DNA analyses. Scale is approximately 1 cm = 85 km. Full names, site codes, and sample sizes for all sites are presented in Table 1.



Genetic Diversity and Differentiation

Results of all analyses suggested slight but significant genetic differentiation of sites. Comparable indicators of differentiation were obtained for both the 1996 and 1997 data sets. UPGMA cluster analysis of individual flycatchers showed unique DNA fingerprints for each individual and only limited clustering of individuals based on site in either 1996 or 1997 (Appendix 2). Flycatchers from the Kern River Preserve (CA) showed the greatest degree of clustering. Results of a site-by-site UPGMA cluster analyses (performed by grouping the flycatchers in each site) on the average between-site inter-observational similarity coefficients are shown in Fig. 2. Branch lengths between sites were relatively long, and generally occurred at similarity values < 0.50. In the correlation analysis of the inter-observational similarity and binary matrices, correlation coefficients were 0.0576 ($p < 0.0005$) and 0.0715 ($p < 0.0005$) for the 1996 and 1997 data, respectively, suggesting that similarity values are on average greater within sites than between sites. Θ-values from each year were 0.1039 (upper C.I. = 0.1353, lower C.I. = 0.0750) and 0.0951 (upper C.I. = 0.1208, lower C.I. = 0.0703) for 1996 and 1997, respectively. Note that confidence intervals for each year are overlapping, suggesting that there is no difference in this statistic between years. Results of the AMOVA analysis are listed in Table 3. Φ_{ST} values were 0.070 ($p<0.0001$) in 1996 and 0.057 ($p<0.0001$) in 1997, indicating that 5.7% and 7.0% of the total genetic variation was a result of the partitioning of individuals into sites.

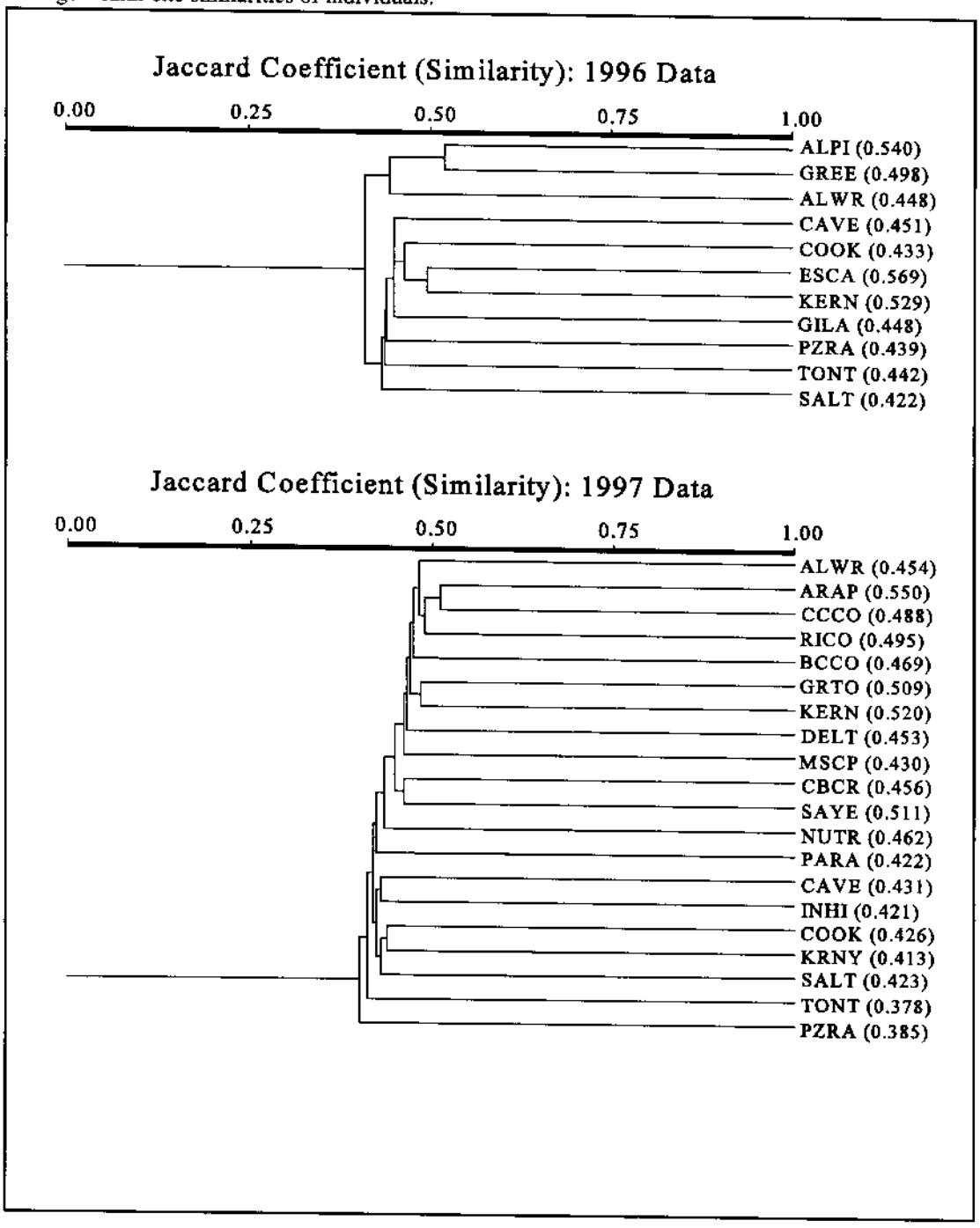
Table 2. Selective nucleotides from each primer combination and their marker profiles. Numbers in parentheses are the polymorphic bands scored in this study.

Restriction site							
EcoRI	AGC for all 6 combinations						
MseI	ACG	ATC	AGC	ACA	ACT	AGG	
Markers							Totals
monomorphic	14	88	105	115	105	84	511
polymorphic	19 (14)	38 (9)	48 (7)	28 (2)	39 (8)	25 (5)	197 (45)
Total	33	126	153	143	144	109	708

Table 3. Results of the AMOVA procedure on data from 1996 and 1997. An asterisk (*) signifies $p<0.0001$.

1996 Data	Source	d.f.	SS	MS	% of total variation
	among sites	9	102.6924	11.410	7.01%*
	within sites	76	530.7146	6.983	92.99%
1997 Data	Source	d.f.	SS	MS	% of total variation
	among sites	18	212.7635	11.820	5.66%*
	within sites	166	1246.0149	7.506	94.34%

Figure 3. UPGMA dendrogram of average between-site genetic similarities (Jaccard Coefficient) of individual willow flycatchers for DNA samples taken in 1996 and 1997. Numbers in parentheses are average within-site similarities of individuals.



In order to determine the effect of sample size and population size on genetic diversity, we performed linear regression analyses of the average heterozygosity and the percent of polymorphic markers versus the estimated number of territories at each site and the number of samples collected at each site. Both measures of genetic diversity increased nonsignificantly with estimated population size (e.g., number of territories) and significantly with sample size (Figure 4). Upon examining the graphical output, one site (Alamosa NWR, CO) appeared to be an outlier in that it had relatively low diversity for a relatively large population size. When we performed a second linear regression that excluded the Alamosa site, the relationship between genetic diversity and estimated population size became significant (Figure 5).

Figure 4. Linear regression of two measures of genetic diversity (average site heterozygosity and the percent of polymorphic markers) with estimated population size (number of territories per site) and sample size per site. Data are from AFLP analysis, 1996 and 1997 combined.

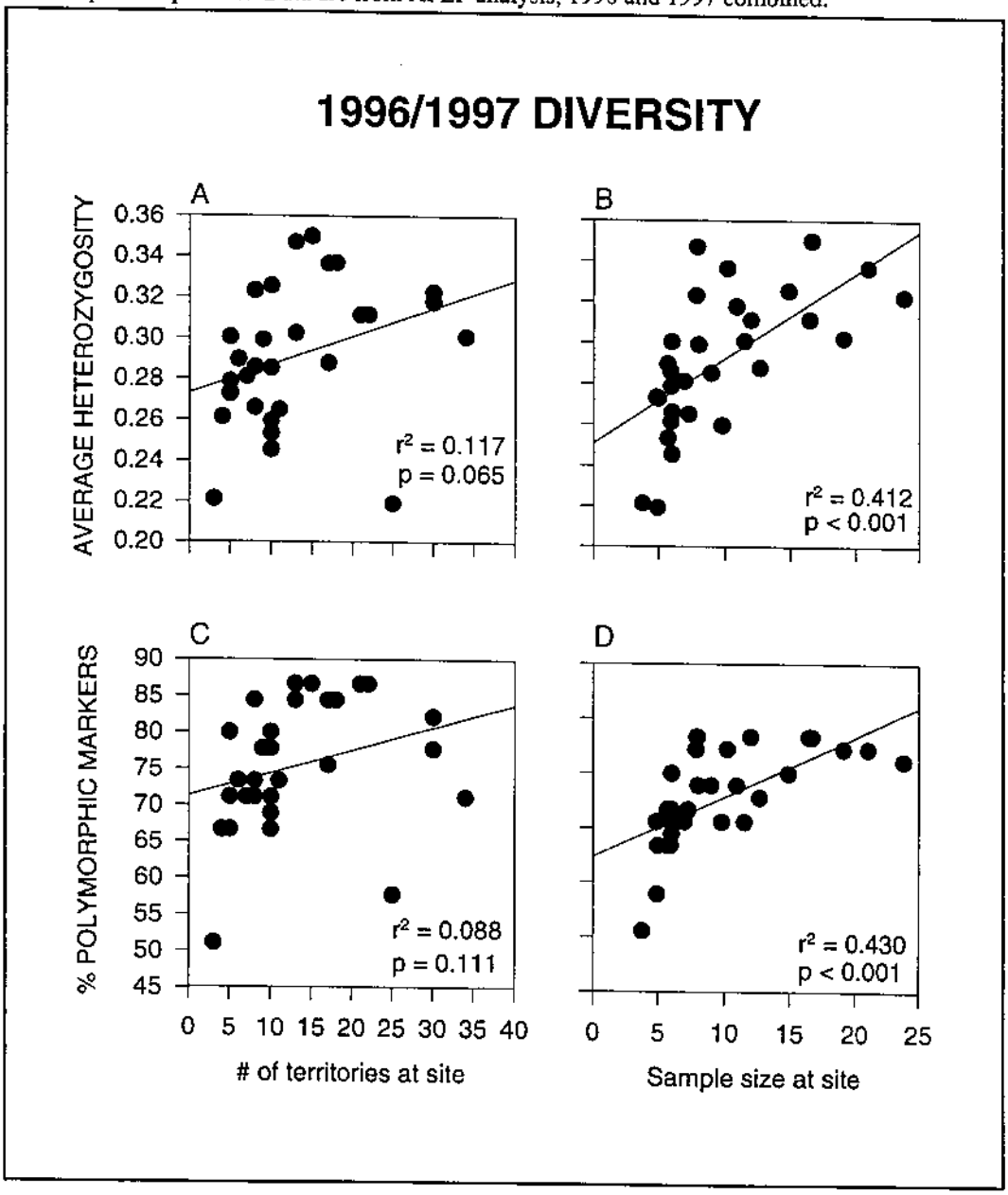
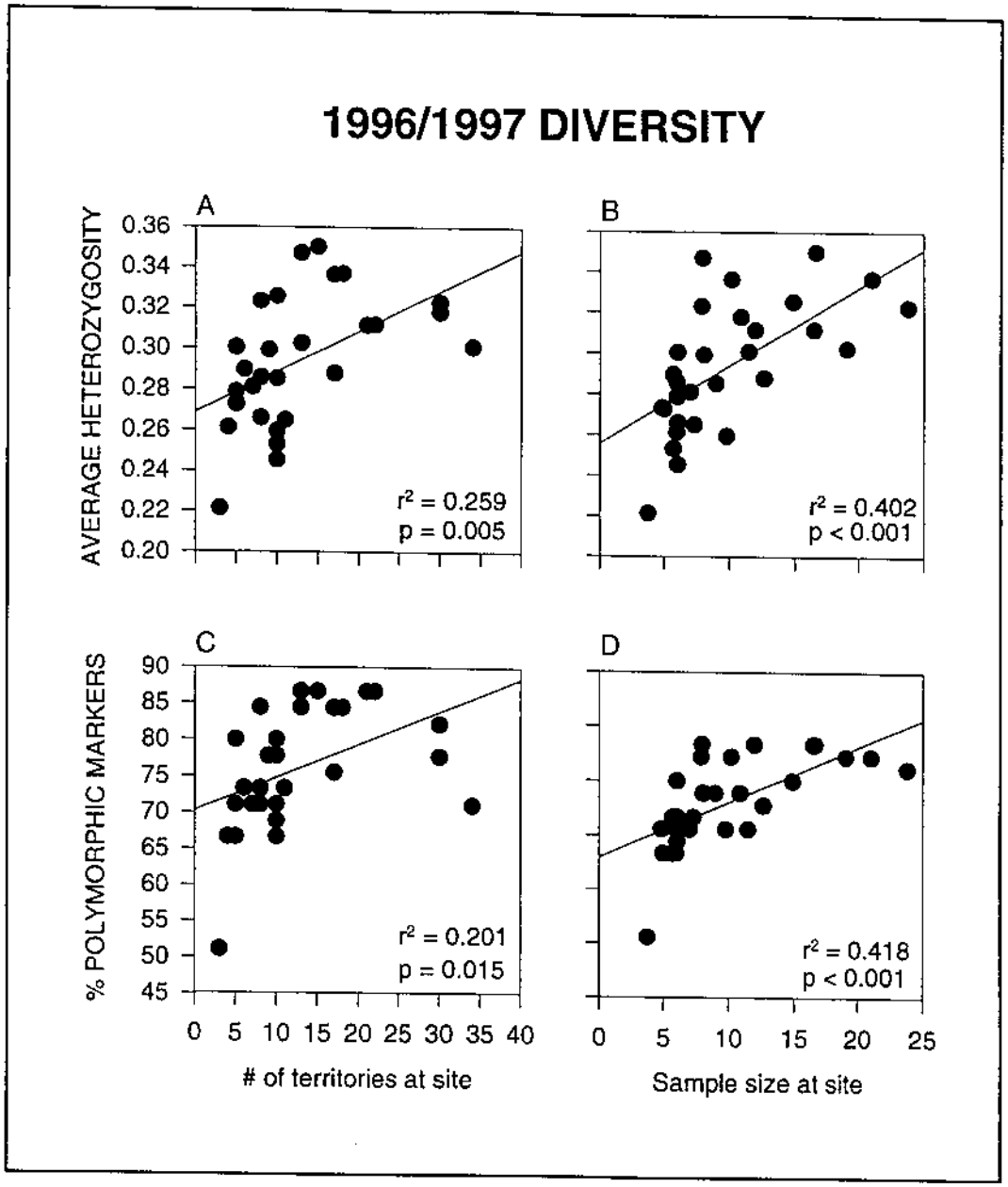


Figure 5. Linear regression of two measures of genetic diversity (average site heterozygosity and the percent of polymorphic markers) with estimated population size (number of territories per site) and sample size per site. Data are from AFLP analysis, 1996 and 1997 combined, excluding the Alamosa NWR site.



Cytochrome-b sequencing

We sequenced the cytochrome-b gene (1143 base pairs) of 20 willow flycatchers from ten sites and seven drainages (Table 4). All willow flycatcher sequences were aligned with the mitochondrial sequence of the chicken (obtained from Genbank) to confirm that the sequence was the cytochrome-b gene. The 20 sequences were then aligned with one another to look for polymorphisms in the form of single nucleotide point mutations.

Two categories of mutations were found. The first category is mutations shared by at least two individuals, which implies common ancestry and were used to place individuals into different mitochondrial linages. Three such lineages (mito-types) were detected (referred to hereafter as types A, B, and C). The second category of mutations included specific nucleotide changes not found in other sampled flycatchers. Such unique mutations were always found within a particular mito-type. These unique mutations were valuable in gauging genetic diversity. Three individuals were found to have a single point mutation falling into this category. Thus, we found three mitochondrial lineages, two of which had more than one haplotype (see Table 4).

The three mitochondrial lineages were separated from one another by one or two nucleotide point mutations, which may indicate a close genetic relationship. Type A is separated from the types B and C by one nucleotide mutation, while types B and C are separated by two mutations (through type A; thus the relationship is C <> A <> B; <> equals a single nucleotide mutation). The unique mutations were also of a single nucleotide change, with the exception of the Grand Canyon flycatcher that was different from its respective mito-type (type B) by two mutations. One of the mutations of this Grand Canyon bird, as well as the mutation that separates type B from type A, resulted in an amino acid change: Valine to Isoleucine and Glutamic Acid to Aspartic Acid, respectively.

Table 4. Mitochondrial lineages and haplotypes of 20 individual willow flycatchers for which the entire cytochrome-B gene was sequenced. Sample location and frequency of a given haplotype at a location are listed.

Lineage	Haplotype	Breeding Site	Haplotype frequency
A	A	Camp Verde	1
		Topock Marsh	1
		Salt River	2
B	B	Alpine	1
	D	Grand Canyon	1
	E	Greer	1
C	C	Tonto Creek	4
		Salt River	2
		Kearny Sewage Ponds	1
		Cook's Lake Seep	1
		Camp Verde	1
		Gila river at Safford	2
		Topock Marsh	1
	F	Tonto Creek	1

DISCUSSION

Background

Genetic patterns are of importance to the conservation of endangered species because habitat fragmentation and destruction (the primary reason for the decline of the southwestern willow flycatcher) produce small, isolated populations of plants and animals. Such small populations tend to lose genetic variation over time, which may increase the probability of population extinction. Thus, understanding the genetic consequences of small population size is important to good management and recovery efforts (Meffe and Carol 1997).

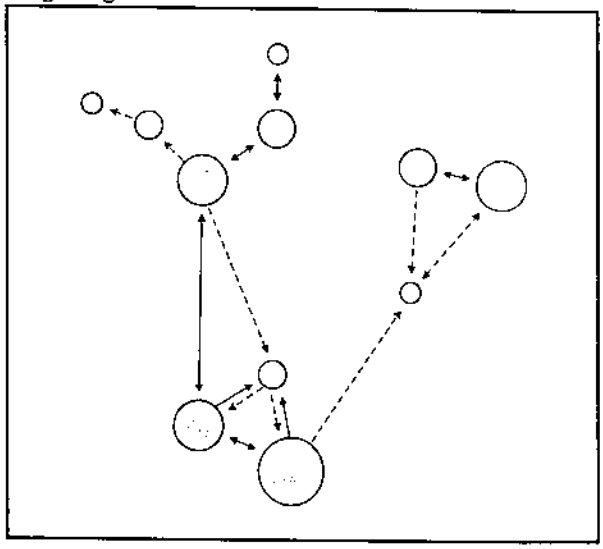
Meffe and Carol (1997) provide an excellent overview of the genetic aspects of conservation biology. We summarize a few of their major points here to provide context for the discussion of our results. The genetic diversity of a species exists at three basic levels; genetic variation within individuals (termed heterozygosity), genetic differences among individuals within a population,and genetic differences among populations (our efforts were focused on characterizing the latter two). Species or subspecies rarely exist as single, randomly interbreeding panmictic populations. Instead, many populations function as members of metapopulations (Figure 6), where a network of populations have some degree of regular or intermittent gene flow among geographically separate units. Thus, there is typically some level of genetic difference among populations that is dependent in large part upon the degree of reproductive isolation in each population. Populations that are relatively isolated and have limited gene flow with other populations typically show higher genetic differentiation, and may have lower levels of within-site genetic variation.

A determination of a species' (or subspecies') within-population versus among-population diversity can define areas of particular conservation interest. For example, a species with low within-population diversity and high among-population differentiation may warrant protection of many populations (each with unique genotypes) in order to protect overall diversity. If a species has low among-population differentiation and high within-population diversity, it may be more important to focus on maintaining the overall population size and protecting highly productive sites, independent of each subpopulation's genome.

The Southwestern Willow Flycatcher

It is critical to remember that the genetic analyses and patterns discussed below do NOT address the question of the subspecies taxonomy of the willow flycatcher, or the geographic boundaries of the different subspecies. The results below, particularly the mitochondrial DNA patterns, will be useful for answering such questions *when additional samples from outside the southwest are*

Figure 6. An illustration of a metapopulation (from Meffe and Carol 1997). Each circle is a local population, with circle size indicative of relative population size. Solid arrows indicate regular and free gene flow; dashed arrows indicate occasional or irregular gene flow.



obtained and analyzed. Currently, we have analyzed samples almost exclusively from within the southwest, and can not predict the nature and degree of genetic variation outside of this area.

AFLP

Our AFLP results suggest that there is substantial genetic variation remaining within the southwestern willow flycatcher breeding groups that we sampled, and that there is only subtle (though statistically significant) genetic differentiation among groups. If there were more limited genetic variation within breeding groups than among breeding groups, UPGMA dendogram branch lengths would be relatively short for individuals and long between groups (Figures 7 and 8). Similarly, individuals of one or more breeding groups with low within-population variation would show substantially shorter branch lengths than individuals of other populations (Figure 9). Limited or no genetic interchange among groups would be noted by all or most individuals from one site clustering together in a UPGMA dendogram (Figures 7-9). In contrast, the individual flycatcher UPGMA dendogram (Appendix 2) shows long branch lengths for individuals and short branches between sites, and individuals from each site are scattered widely across the dendogram and usually loosely grouped with flycatchers from other sites. Similarly, the site-based UPGMA diagram for flycatchers (Figure 3) shows long branch lengths for each site and short branch lengths between sites.

The slight but statistically significant genetic structuring that we did find could arise because most individual flycatchers show some degree of fidelity to their breeding site or general area (Paxton et al. 1997). Although between-site movements regularly occur, there is apparently not sufficient movement to bring about a truly panmictic population which would show no genetic structuring.

These AFLP patterns demonstrate that the southwestern willow flycatcher population is functioning as a metapopulation, with genetic exchange occurring among the different breeding groups. Individuals are dispersing and moving among breeding sites, and genetic data suggest it happening on a regular basis. This is further supported by observations of within- and between year movements of banded flycatchers among different breeding sites, both within the same river drainage (Paxton et al. 1997) and among drainages (USGS, unpublished data).

The level of genetic diversity per site was significantly related to both the estimated population size and the number of samples we collected per site (Figures 4 and 5). The only exception was the Alamosa NWR site, which had lower diversity than would be expected given its estimated population size. Our flycatcher results concur with current population genetics theory which predicts that, all else being equal, smaller populations (or subpopulations) will tend to have lower diversity than larger populations.

Figure 7. A UPGMA cluster analaysis of *Rana pipiens* (leopard frog) populations, from Kimberling et al. 1996. Note that all individuals from each site cluster together, and that genetic distance between sites is much larger than that among individuals within a site. Also note the overall low genetic diversity (compared to Figure 3), and that some populations (such as Buckskin Tank and Hess tank) show much less within-site diversity than other populations (Butch Tank and Stoneman).

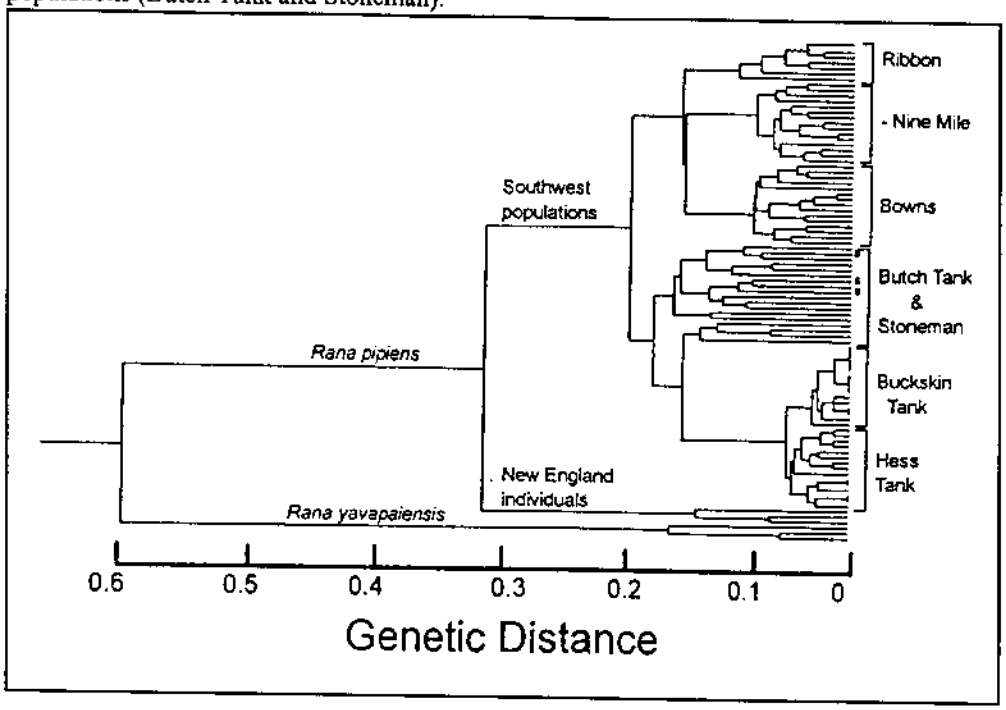


Figure 8. A UPGMA dendogram of similarity coefficients for AFLP markers from four populations of *Oxyloma* snails (from Miller et al. 1998). Note that all individuals of each population cluster together. Also note the overall high genetic similarity (and thus low diversity) within each population, and that the Vasey's Paradise and -9 mile populations have substantially lower diversity than the other two populations.

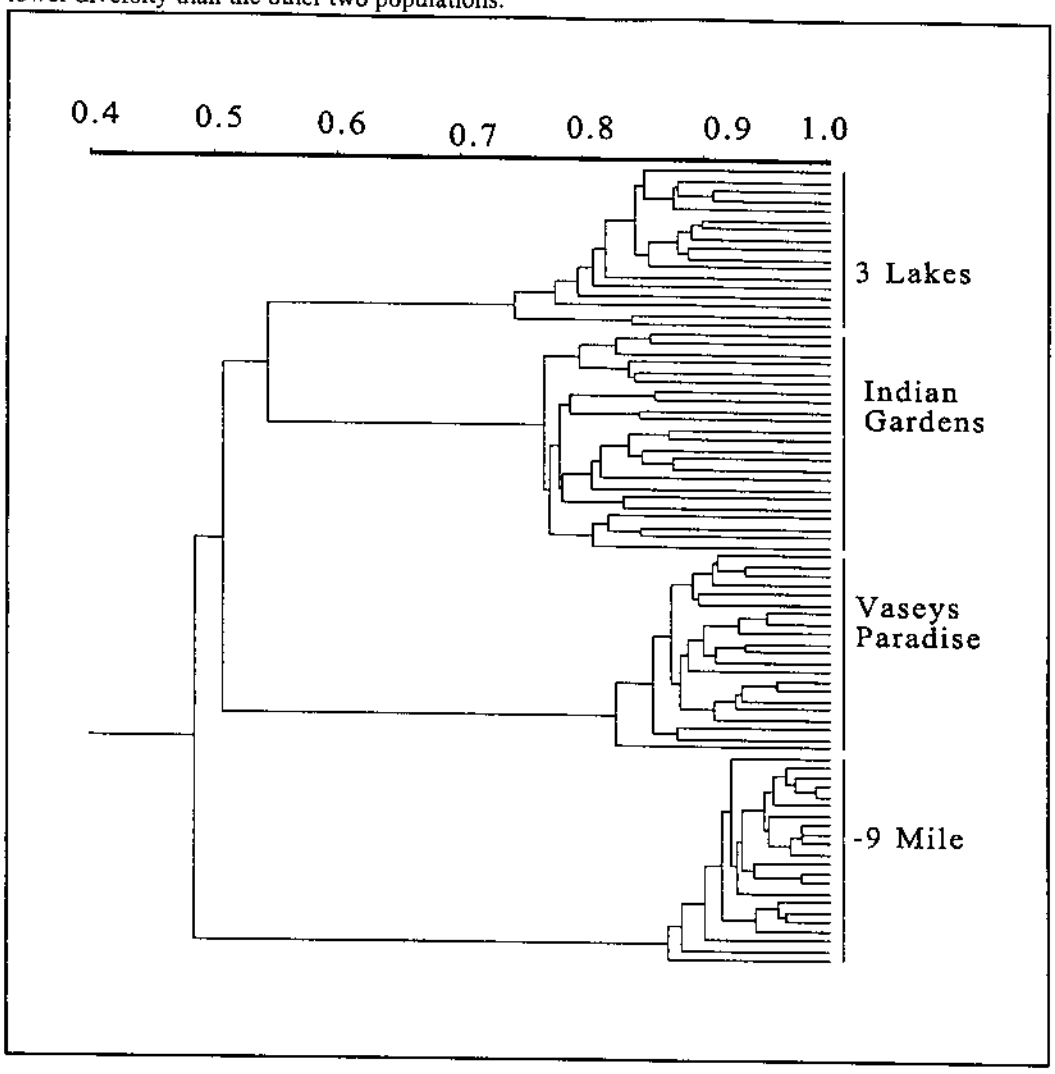
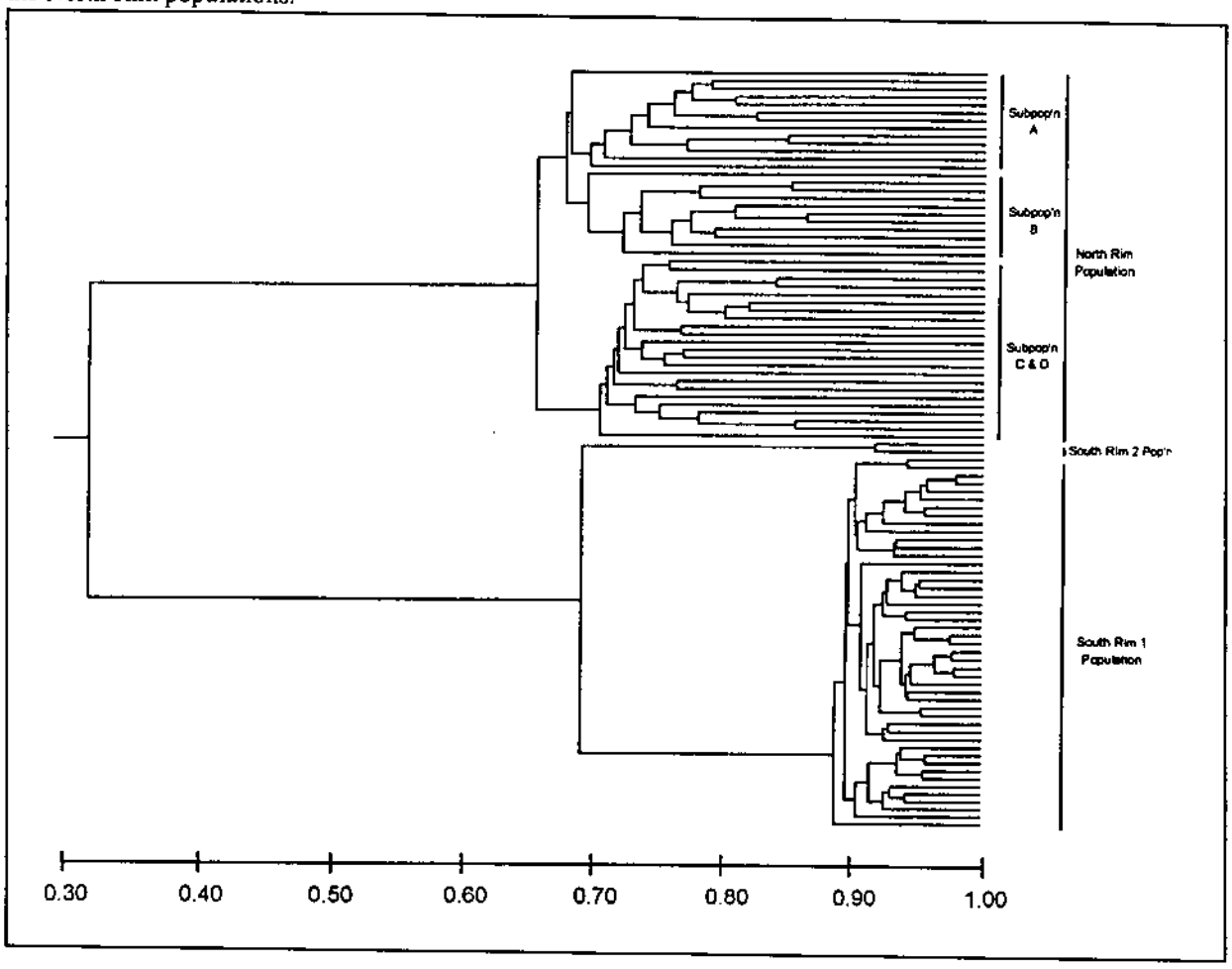


Figure 9. A UPGMA cluster analysis of similarity Dice coefficients based on AFLP analysis of three populations of the endangered plant *Astragalus cremnophylax* var *cremnophylax* in Grand Canyon National Park (from Travis et al. 1996). Note that all individuals from each subpopulation cluster together. Note also the high similarity (and thus very low diversity) within the South Rim populations, which are geographically and reproductively isolated from the North Rim populations.



Mitochondrial DNA

Mitochondrial DNA, including the cytochrome-b gene, moves differently within a population than nuclear DNA, and as such offers an independent analysis of the flycatcher's genetic history for comparison with the AFLP analysis. In addition, mitochondrial DNA is passed down identically from mother to offspring so that it can show female gene flow patterns and allow detection of different patterns of gender-related gene flow. Studies combining both types of analysis produce the strongest results.

The initial survey across Arizona yielded only the three mitochondrial groups, each of which varied by only one or two nucleotides. This may imply closely related groups with high gene flow among sites. Both type A and type C were found throughout the state, while type B was limited to the White Mountains and the Grand Canyon. Such mito-type differences and distributions can not be properly interpreted without further sampling and analysis of DNA sequences throughout the species' range.

The single nucleotide mutation separating each mitochondrial group may represent a recent divergence between the groups. Mitochondrial type A appears to be the ancestral type, as there is only one mutation separating type A with types B and C, but two mutational steps separating types B and C. Only one of the point mutations separating the groups resulted in an amino acid change, the mutation separating type A from type B. All other mutations were silent mutations (mutations that do not change the amino acid being coded for) and thus not under selection pressure; these mutations are considered to occur with much greater frequency than non-silent mutations. However, while the mutation separating type B changes the encoded amino acid, the change is to a very similar amino acids that may not change the chemistry or structure of the protein. Thus this mutation may not be any more significant than a true silent mutation.

We then sequenced 9 birds at Roosevelt Lake to look at within-site diversity. Five were sampled from the Tonto Creek inflow and 4 from the Salt River inflow. Seven of the nine flycatchers had mitochondrial lineage type C, and two had type A (Table 4). Both mito-types were found at the Salt River inflow, but only type C was present at the Tonto Creek inflow. The presence of two mito-types within one breeding site suggests that the two types are not reproductively isolated.

Overall, the mitochondrial analysis appears to support the AFLP-based analysis, suggesting some differentiation (e.g., three different mitochondrial types), but no strong genetic structuring (e.g., two mito-types present at one breeding site). The next step to understanding the significance of these findings will be to compare the Arizona patterns with samples from outside the state. Only then can we determine: (1) the geographic distribution of mitochondrial groups, and (2) if the level of diversity within *E.t. extimus* is similar to that in other subspecies or populations that have not had as great a range restriction and possible bottleneck event in the past.

Management Implications

Our genetic analysis did not reveal any substantially unique genotypes, highly unique breeding groups, or populations with severely limited genetic variation. Thus, we did not find any individual breeding sites that are of special genetic significance or concern. We have not sampled from all known southwestern willow flycatcher breeding groups, and thus can not be sure that genetically unique or isolated populations do not exist. However, given that several of the sites included in our analysis are relatively geographically isolated and/or very small (e.g., Pahranagat, Alpine, Greer, Kern River) yet had high diversity and little differentiation, we believe that few if any of the sites we have not sampled are likely to be substantially differentiated or have low diversity. However, particular sites may be of conservation or management concern for other reasons (see below).

The movements of individuals among breeding populations that maintain this within-site diversity and metapopulation condition may depend upon the existence of a number of sites geographically scattered across the bird's range. Current banding data (Paxton et al. 1997, USGS unpublished data) suggest that movement is more frequent among geographically closer than more distant sites. Thus, maintenance of a network breeding sites close enough to allow for regular dispersal of individuals, and thus genetic exchange, is crucial if one wished to avoid a metapopulation pattern where subpopulations begin to experience limited genetic variation and high differentiation among populations.

Because no subpopulations show substantial genetic divergence and genetic exchange is occurring among sites, the major conservation focus should be on maintaining and increasing the overall willow flycatcher population in the southwest. A related consideration is the protection of flycatcher breeding areas with high productivity and breeding success. Such "source" populations (Pulliam 1988) increase the number of individuals in the overall population (hence reducing the potential for extinction), and the number available for dispersal among sites (which increases genetic interchange and maintains genetic diversity).

Acknowledgments

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Literature Cited

Avise, J.C. 1994. Molecular markers, natural history, and evolution. Chapman and Hall, New York.

Browning, M.R. 1993. Comments on the taxonomy of *Empidonax traillii* (willow flycatcher). Western Birds 24:241-257.

Cooper, C. A. 1997. Statewide summary of 1996 surveys for Willow Flycatchers in New Mexico. Final Report: Contract #96-516.81. New Mexico Department of Game and Fish: Santa Fe, New Mexico.

Excoffier, L., P.E. Smouse and J.M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479-491.

Excoffier, L. 1993. AMOVA version 1.55. Computer software distributed by author.

Helm-Bychowski, K. and J. Cracraft. 1993. Recovering phylogenetic signal from DNA sequences: relationships within the Corvine assemblages (Class Aves) as inferred from complete sequences of the mitochondrial DNA cytochrome-b gene. Molecular Biology and Evolution 10:1196-1214.

Hoelzel, A.R. and A. Green. 1992. Analysis of population-level variation by sequencing PCR-amplified DNA. Pp 159-188 *in* Molecular Genetic Analysis of Populations, A.R. Hoelzel, *ed.*, Oxford University Press. 315 pp.

Hubbard, J. P. 1987. The status of the willow flycatcher in New Mexico. Endangered Species Program, New Mexico Department of Game and Fish, Santa Fe, New Mexico. 29 pp.

Jaccard P. 1908. Nouvelles recherches sur la distribution florale. Bull. Soc. Vaud. Sci. Nat. 44: 223-270.

Kimberling, D.N., A.R. Ferreira, S.M. Shuster and P. Keim. 1996. RAPD marker estimation of genetic structure among isolated northern leopard frog populations in the south-western USA. Molecular Ecology 5:521-529.

Lande, R. 1988. Genetics and demography in biological conservation. Science 241:1455-1460.

Langridge, S.M. and M.K. Sogge. 1997. Banding of the Southwestern Willow Flycatcher in the White Mountains. USGS Colorado Plateau Research Station: Flagstaff, Arizona.

Langridge, S. M. and M. K. Sogge. 1997b. Banding and population genetics of the Southwestern Willow Flycatcher in Zuni Land - 1997 summary report. USGS Colorado Plateau Research Station: Flagstaff, Arizona.

Lynch, M. and B.G. Milligan. 1994. Analysis of population genetic structure with RAPD markers. Molecular Ecology 3:91-99.

Mantel, N. 1967. The detection of disease clustering and a generalized regression approach. Cancer Research 27: 209-220.

Marshall, R.M. *In review.* Current status and distribution *in* Southwestern Willow Flycatcher Conservation Assessment. U.S.D.A. Forest Service, Rocky Mountain Range and Experimental Station General Technical Report.

Maynard, W.R. 1995. Summary of 1994 survey efforts in New Mexico for Southwestern Willow Flycatcher (*Empidonax traillii extimus*). New Mexico Game and Fish Department, Santa Fe.48 pp.

Meffe, G.K. and C.R. Carroll. 1997. Principles of Conservation Biology. Sinauer Associates, Inc., Sunderland, Massachusetts.

Miller, M.P. *in press*. MANTEL-STRUCT: A program for the detection of population structure via Mantel tests. Journal of Heredity.

Miller, M.P. 1997. Tools for Population Genetic Analyses (TFPGA) version 1.3: A Windows program for the analysis of allozyme and molecular population genetic data. Computer software distributed by author.

Miller, M.P. 1997. AMOVA-PREP version 1.01: A program for the preparation of AMOVA input files from dominant-marker raw data. Computer software distributed by author.

Miller, M.P., J. Busch, J. Sorenson, L.Stevens, and P. Keim. 1998. Genetic diversity, population structure, and relationships of the Kanab Amber Snail *(Oxyloma haydeni haydeni)* in the southwest USA. Report to the National Park Service and Arizona Game and Fish Department.

Mullenbach, R., J.P.L. Lagoda, and C. Welter. 1989. An efficient salt-chloroform extraction of DNA from blood and tissues. Trends in Genetics 5:391.

Nevo, E. 1978. Genetic variation in natural populations: patterns and theory. Theoretical Population Biology 13:121-177.

Owen, J. C. and M. K. Sogge. 1997. Banding and Genetic Sampling of Willow Flycatchers in Colorado - 1996 & 1997 Summary Report. USGS Colorado Plateau Field Station: Flagstaff, Arizona.

Paxton, E. and M. K. Sogge. 1996. Banding and population genetics of Southwestern Willow Flycatchers in Arizona: 1996 Summary Report. USGS, Colorado Plateau Research Station: Flagstaff, Arizona.

Paxton, E. H., S. M. Langridge, and M. K. Sogge. 1997. Banding and Population Genetics of Southwestern Willow Flycatchers in Arizona - 1997 Summary Report. USGS Colorado Plateau Field Station: Flagstaff, Arizona.

Pulliam, H.R. 1988. Sources, sinks, and population regulation. American Naturalist 132:652-661.

Ralph, C.J., G.R. Geupel, P. Pyle, T.E. Martin and D.F. DeSante. 1993. Handbook of Field Methods for Monitoring Landbirds. USDA Forest Service General Technical Report PSW-GTR-144.

Rohlf, F.J. 1993. NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System, version1.8. Exeter Software, Setauket, NY.

Seitz, A. And V. Loeschcke (eds.). 1991. Species conservation: a population-biological approach. Birkhauser Verlag, Basel.

Spencer, J.A., S.J. Sferra, T. E. Corman, J. W. Rourke, J. A. Spencer, and M. W. Sumner. 1996. Arizona Partners in Flight 1995 Southwestern Willow Flycatcher Survey. Arizona Game and Fish Department: Phoenix, Arizona. Nongame and Endangered Wildlife Program Technical Report 97.

Sferra, S. J., T. E. Corman, C. E. Paradzick, J. W. Rourke, J. A. Spencer, and M. W. Sumner. 1997. Arizona Partners in Flight Southwestern Willow Flycatcher Survey: 1993-1996 Summary Report. Arizona Game and Fish Department: Phoenix, Arizona. Nongame and Endangered Wildlife Program Technical Report 113.

Sogge, M. K., R. M. Marshall, S. J. Sferra, and T. J. Tibbitts. 1997. A Southwestern Willow Flycatcher Natural History Summary and Survey Protocol. Colorado Plateau Research Station, Northern Arizona University: Flagstaff, Arizona. National Park Service Technical Report USGS/NAUCPRS/NRTR-97/12.

Sokal R and Rohlf FJ, 1995. Biometry, 3rd edition. New York, New York: W.H. Freeman.

Travis, S.E., J. Maschinski, and P. Keim. 1996. An analysis of genetic variation in *Astragalus cremnophylax* var. *cremnophylax*, a critically endangered plant, using AFLP markers. Molecular Ecology 5:735-745.

Unitt, P. 1987. *Empidonax traillii extimus*: An endangered subspecies. Western Birds 18:137-162.

U.S. Fish and Wildlife Service. 1993. Proposal to list the southwestern willow flycatcher as an endangered species and to designate critical habitat. Fed. Reg. 58:39495-39522 (July 23, 1993).

U.S. Fish and Wildlife Service. 1995. Final Rule Determining Endangered Status for the Southwestern Willow Flycatcher. Federal Register 60:10694 (February 27, 1995).

U.S. Fish and Wildlife Service. 1997. Final Biological Opinion regarding long-term operation of Isabella Reservoir. April 18, 1997. Ecological Services, Sacramento Field Office.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research. 23:4407-4414.

Weins, D., D.L. Nickrent, C.I. Davern, C.L. Calvin and N.J. Vivrette. 1989. Developmental failure and loss of reproductive capacity in the rare paleoendemic shrub *Dedeckera eurekensis*. Nature 338:65-67.

Weir, B.S. 1996. Genetic Data Analysis II: Methods for Discrete PopulationGenetic Data. Sinauer Associates, Inc. Sunderland, Massachusetts.

Weir, B.S. and C.C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. Evolution 38(6):1358-1370.

Whitfield, M. J. and K. M. Enos. 1996. A Brown-headed Cowbird control program and monitoring for the Southwestern Willow Flycatcher, South Fork Kern River, California, 1996. Final report to the U.S. Army Corps of Engineers, Contract DACW05-96-P-0900. Kern River Research Center: Weldon, California.

Whitfield, M.J., K.M. Enos, and S.P. Rowe. 1997. Reproductive Response of the Southwestern Willow Flycatcher (*Empidonax traillii extimus*), to the removal of Brown-headed Cowbirds. DRAFT Admin. Rept. To ACOE.

Zink, R.M., S. Rohwer, A.V. Andreev, and D.L. Dittmann. 1995. Trans-Beringia comparisons of mitochondrial DNA differentiation in birds. Condor 97:639-649.

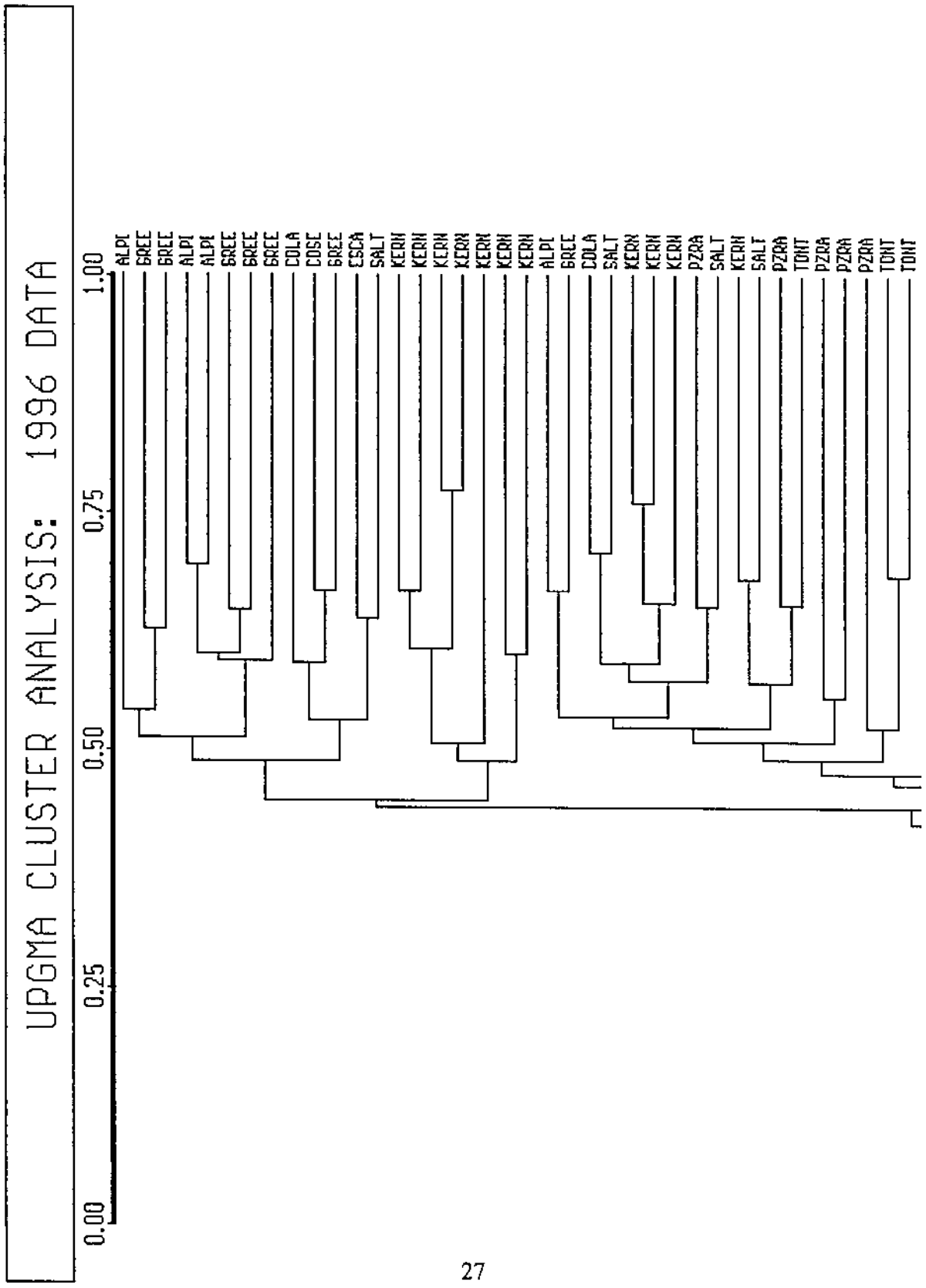
Appendix 1. The estimated number of breeding territories at each of the willow flycatcher breeding sites used for AFLP analysis.

Appendix 1. The estimated number of breeding territories at each of the willow flycatcher breeding sites used for AFLP analysis. Estimates are based on Spencer et al. 1996, Whitfield and Enos 1996, Owen and Sogge 1997, Sferra et al. 1997, Whitfield et al. 1997, and USFWS unpublished data.

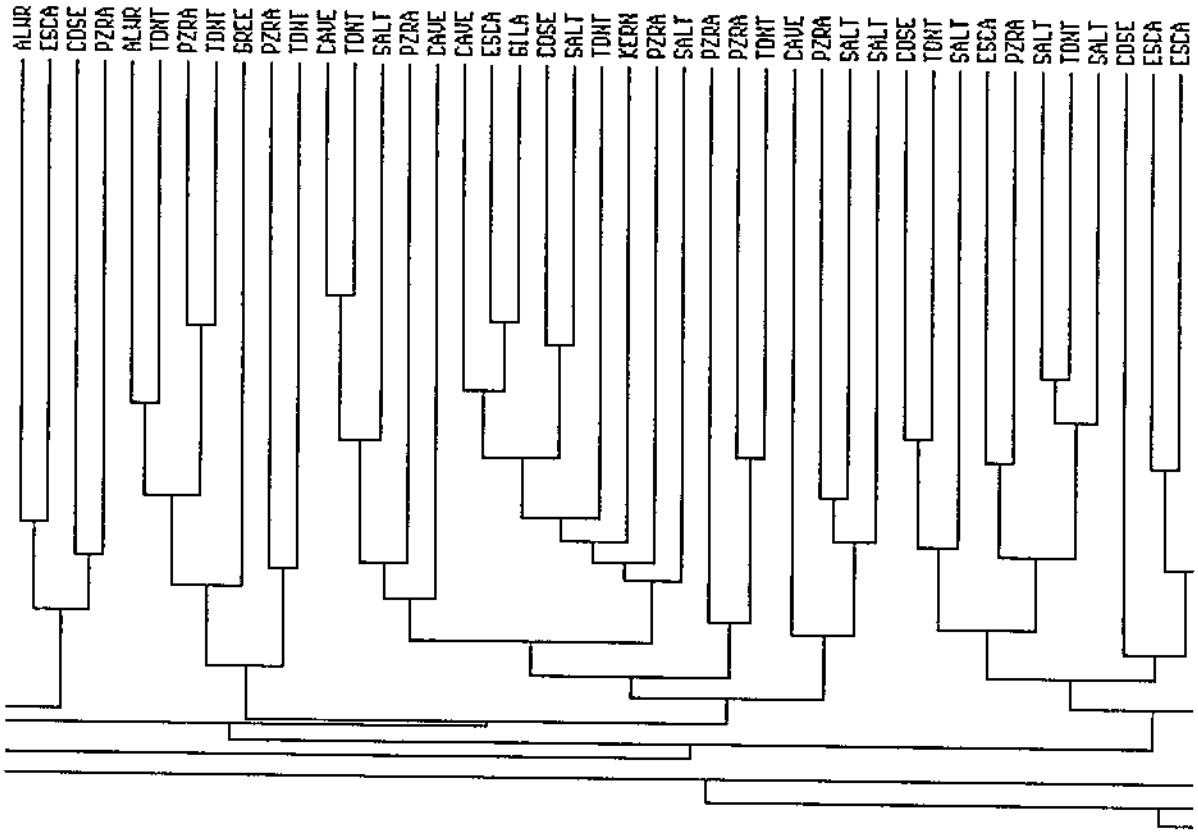
Estimated "population" size per site	Site code	Estimated number of territories at site	
		1996	1997
Alpine, AZ	ALPI	3	
Alamosa NWR, CO	ALWR	30	30
Beaver Creek, CO	BCCO		10
Camp Verde, Verde River, AZ	CAVE	6	10
CB Crossing, San Pedro Riv, AZ	CBCR		5
Clear Creek, CO	CCCO		9
Cook's Lake, San Pedro River, AZ	COOK	17	13
Escalante State Wild. Ref., CO	ESCA	10	8
Gila River at Safford, AZ	GILA	4	
Greer, AZ	GREE	11	7
Indian Hills, San Pedro River, AZ	INHI		15
Kern River Preserve, CA	KERN	34	30
Kearny, Gila River, AZ	KRNY		8
McIntyre Springs, CO	MCSP		8
Pahranagat NWR, NV	PARA		5
PZ Ranch, San Pedro River, AZ	PZRA	13	5
Rio Blanco, CO	RICO		10
Salt River Inflow, Roosevelt Lk, AZ	SALT	22	18
Santa Ynez River, near Buelton, CA	SAYE		10
Tonto Creek Inflow, Roosevelt Lk, AZ	TONT	17	21
Zuni Pueblo, NM	ZUNI		5

Appendix 2. UPGMA dendograms of similarity coefficients for AFLP analysis of DNA samples from individual willow flycatchers captured and blood-sampled in 1996 and 1997.

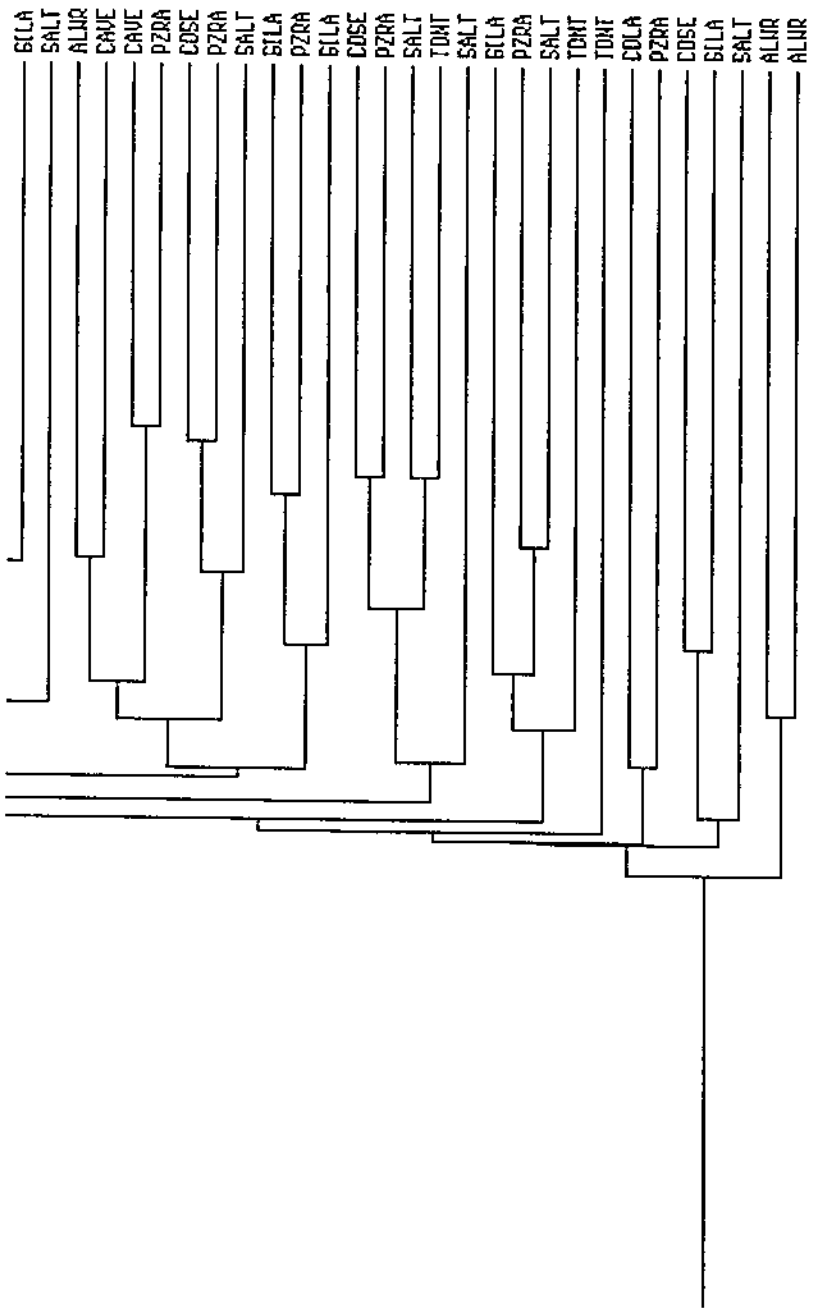
Appendix 2a. UPGMA dendogram of similarity coefficients for AFLP analysis of DNA samples from individual willow flycatchers at 11 sites during 1996. The full names for site codes are given in Appendix 2.



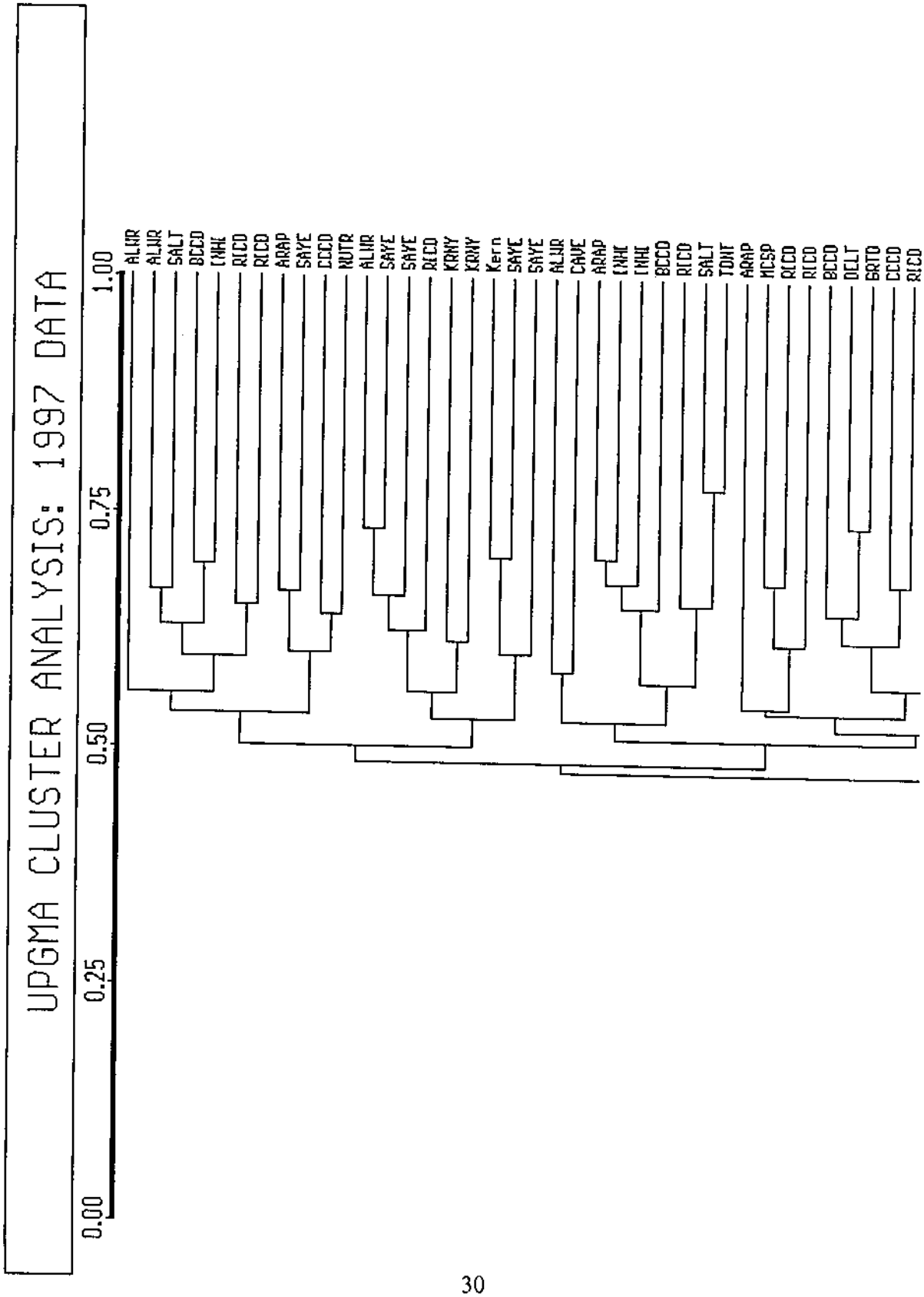
Appendix 2a continued. UPGMA dendogram of similarity coefficients for AFLP analysis of DNA samples from individual willow flycatchers at 11 sites during 1996. The full names for site codes are given in Appendix 2.



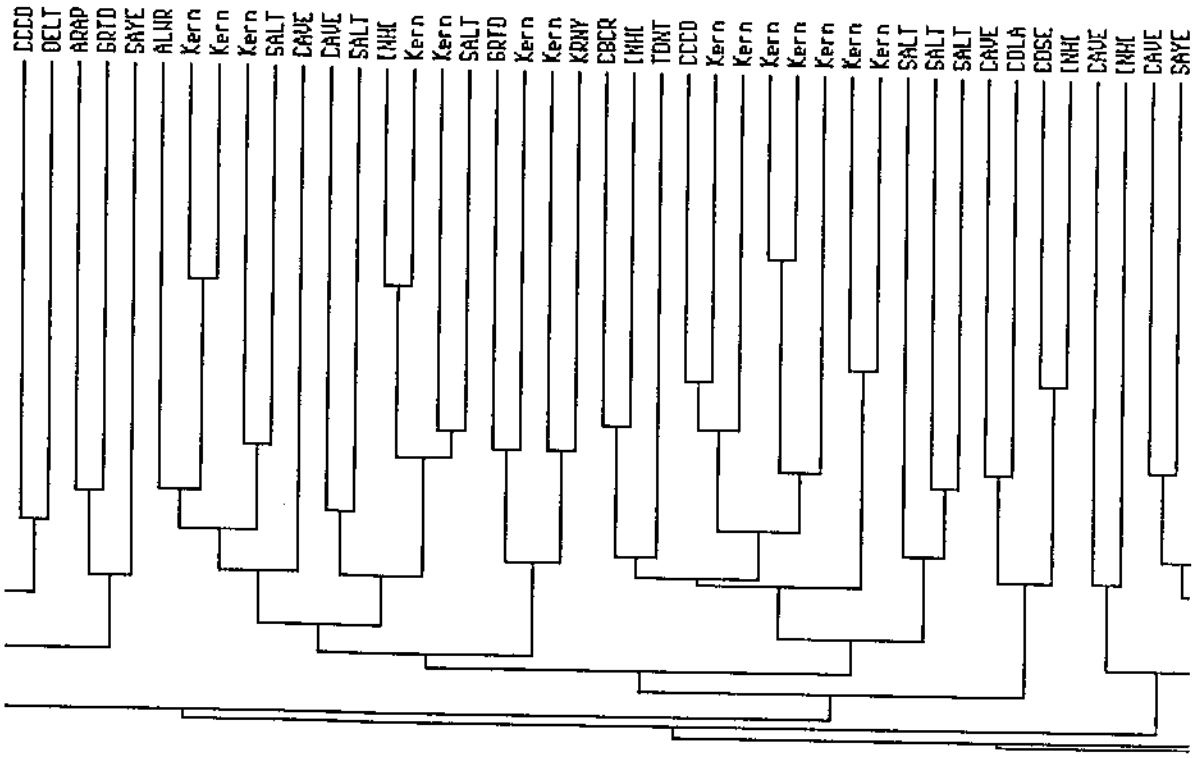
Appendix 2a continued. UPGMA dendogram of similarity coefficients for AFLP analysis of DNA samples from individual willow flycatchers at 11 sites during 1996. The full names for site codes are given in Appendix 2.



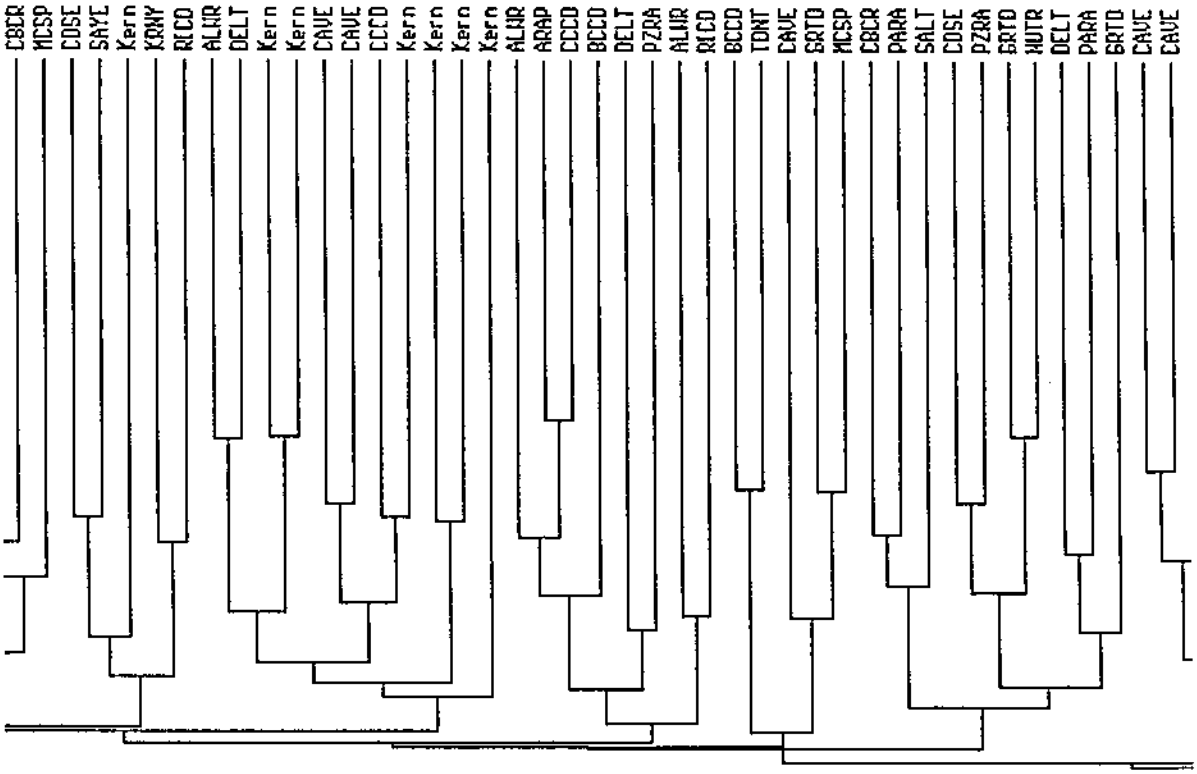
Appendix 2b. UPGMA dendogram of similarity coefficients for AFLP analysis of DNA samples from individual willow flycatchers at 19 sites during 1997. The full names for site codes are given in Appendix 2.



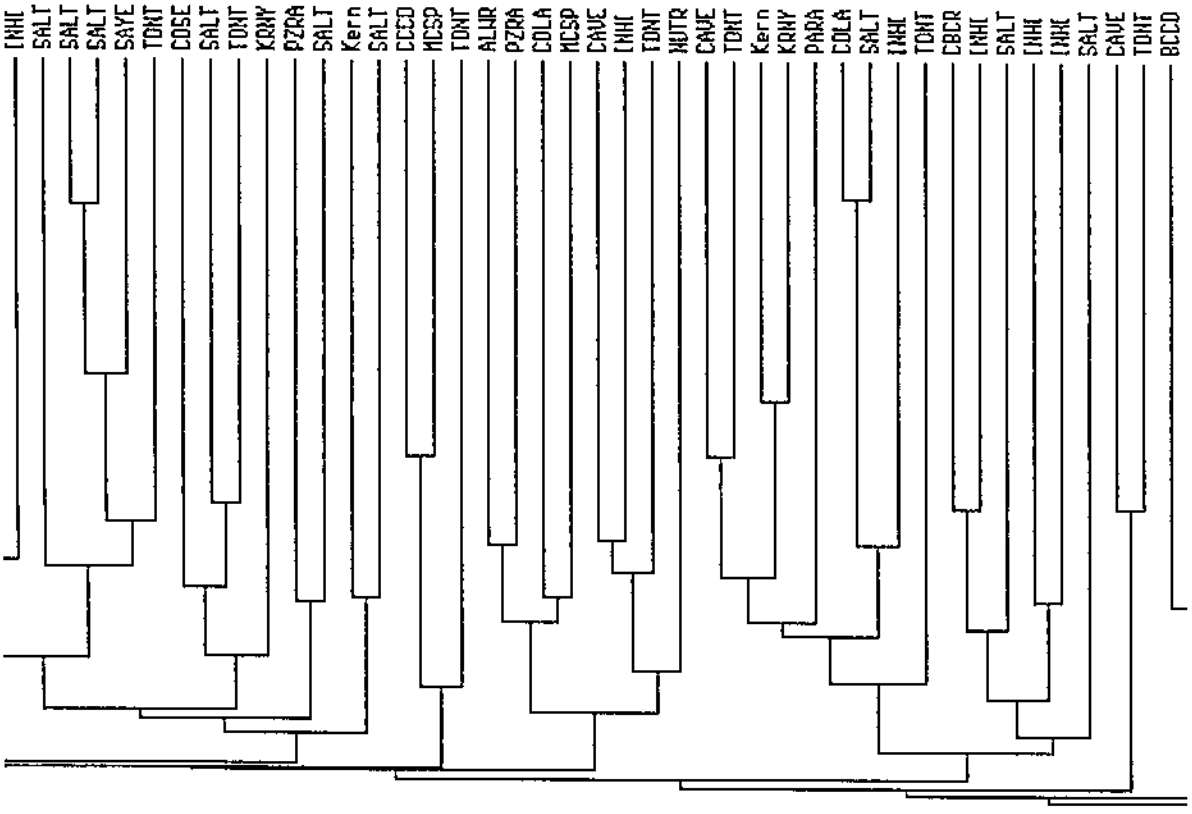
Appendix 2b continued. UPGMA dendogram of similarity coefficients for AFLP analysis of DNA samples from individual willow flycatchers at 19 sites during 1997. The full names for site codes are given in Appendix 2.



Appendix 2b continued. UPGMA dendogram of similarity coefficients for AFLP analysis of DNA samples from individual willow flycatchers at 19 sites during 1997. The full names for site codes are given in Appendix 2.



Appendix 2b continued. UPGMA dendogram of similarity coefficients for AFLP analysis of DNA samples from individual willow flycatchers at 19 sites during 1997. The full names for site codes are given in Appendix 2.



Appendix 2b continued. UPGMA dendogram of similarity coefficients for AFLP analysis of DNA samples from individual willow flycatchers at 19 sites during 1997. The full names for site codes are given in Appendix 2.